A role for cell migration in the sexual transmission of HIV-1?

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The issue of how human immunodeficiency virus-1 (HIV-1) enters the body following sexual contact has been the subject of considerable controversy. Several possible routes for the initial infection have been suggested [1–6], including the possibility that the transmission is mediated by HIV-1-infected lymphocytes or macrophages in serum and female genital tract secretions, rather than by free virus. We recently reported that HIV-1-infected, activated primary monocytes can migrate between epithelial cells grown in confluent monolayer cultures *in vitro* [7]. We report here on experiments carried out in mice to test the hypothesis that mononuclear blood cells are capable of migrating through intact epithelia, and thus of carrying a virus into an animal. We placed double-stained, activated mononuclear blood cells into the vaginas of mice; four hours later, numerous double-stained cells were observed in the connective tissue beneath the vaginal epithelium and the iliac lymph nodes of the experimental mice. We speculate that such migration may be involved in the sexual transmission of HIV-1.

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Results and discussion

In order to detect mononuclear blood cells that may have migrated from the vagina, we inoculated female BALB/c mice intraperitoneally with Brewer's thioglycolate broth (Difco, Detroit), a procedure which is commonly used to recruit differentiated macrophages [8]. Peritoneal cells were collected three days later by abdominal lavage with 5 mL of RPMI 1640, and then washed in phosphate-buffered saline (PBS) and divided into three parts for immunophenotyping.

Immunophenotyping was carried out by labeling the cells, after pre-incubation in rat immunoglobulin G (IgG), with the following fluoroscein-isothiocyanate (FITC)-labelled monoclonal antibodies: anti-F4/80 (Serotec, Oxford), a marker for resident and elicited macrophages [9]; anti-CD3 (clone 29B), a pan-T cell marker (Sigma, St Louis); and anti-CD45R (clone RA3-6B2), a B-cell marker (Sigma, St Louis). We determined that 85–95% of the cells were positive for anti-F4/80, 3–11% were positive for anti-CD3, and 2–3% were positive for anti-CD45R. This showed that most of the peritoneal cells we collected were macrophages, as others have observed using similar procedures [8].

We stained peritoneal mononuclear cells with supravital dyes that are known to be non-cytotoxic and have been previously shown to be suitable for following cell migration [10–12]. We used two supravital dyes so that the cells could be identified with greater confidence: Hoechst 33342, a blue fluorochrome that binds DNA in the nucleus, and PKH26-GL, a red fluorescent probe that is incorporated into the plasma membrane. The peritoneal cells were counted, and 2×10^7 cells were resuspended in 1 mL of Diluent C provided in the PKH26-GL staining kit (Sigma, St Louis). Cells were then mixed with 1 mL of 4×10^{-6} M PKH26-GL dye, according to the manufacturer's instructions, and washed four times in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Subsequently, the cells were resuspended in PBS containing 3 μg mL^{-1} Hoechst 33342 (bisbenzimide, Sigma, St Louis) and incubated in the dark for 30 minutes at room temperature with occasional mixing. All cells were found to be stained by both dyes.

Recipient 8 week-old female mice, of either the BALB/c or outbred CD1 strain, were injected subcutaneously with 100 μL of a 25 mg ml^{-1} Depo Provera solution (Upjohn, Kalamazoo) in PBS. Five days after progestin treatment, 4×10^6 double-stained cells in 25 μL of RPMI 1640 with 10% FBS were inoculated into the vaginas of sedated mice, using a P-20 Pipetman (Rainin, Woburn) fitted with a pipet tip, with or without a silastic tube placed over it. Four hours later, mice were sacrificed and the iliac lymph nodes and vaginas were removed; the vaginas were embedded in OCT compound (Miles Inc., Ekhart) and frozen.

We examined lymph nodes, because it has been well documented that mononuclear cells traffic from mucosal surfaces to nearby lymph nodes [13]. Cells were extracted from lymph nodes by mincing and passing the minced tissue through a 70 μm mesh insert (Falcon, Becton Dickenson, Lincoln Park) [14]. Lymph node cells were washed in PBS at 400 x *g* for 5 minutes, and the cells were counted. They were then fixed in 2% paraformaldehyde for 30 minutes and washed twice in PBS. The cells were resuspended in 20 μL of PBS, and 4 μL aliquots were spotted onto 8 ring slides. The slides were sealed with

clear nail polish before viewing in a Nikon epifluorescence microscope equipped with an Omega cube. Cells that had a red cytoplasmic stain and blue nuclei were counted. Additional experiments were carried out to determine the immunophenotype of the mononuclear cells that had reached the lymph nodes. For each of five animals, half the lymph node cells were stained with an FITC-labeled monoclonal antibody against the macrophage marker F4/80, and half with an FITC-labeled monoclonal antibody against the pan-T cell marker CD3.

The vaginas that had been embedded in OCT and frozen were cut into 10 μm serial sections in a cryostat and air dried. The sections were then fixed in 2% paraformaldehyde for 20 minutes, washed twice in PBS and mounted using an aqueous mounting medium. To determine if the inoculation of cells had produced trauma, serial sections of vaginas were examined with phase microscopy. No evidence of trauma was seen. The sections were examined to determine if cells had migrated from the vaginal vault to the connective tissue below the epithelium. Numerous double-stained cells were observed in the connective tissue (lamina propria) below the vaginal epithelium in each of the eight experimental animals (Fig. 1a,c). In a number of cases, several cells were seen in the same section (Fig. 1e). To be confident that fluorescence was not due to autofluorescence or leakage of dye, we also examined sections from the vaginas of two uninoculated, progestin-treated mice and two animals which had been inoculated with freeze-thawed cells; no double-stained cells were seen in tissues of these animals.

We viewed cells from the dissociated iliac lymph nodes of the six inoculated and four control animals. Double-stained cells were easily distinguishable in the inoculated mice (Fig. 1g). The experimental animals had an average of about 45 cells each, whereas no double-stained cells were seen among the lymph node cells of the four control mice (Table 1). An additional five animals were used to identify the phenotype of cells that had reached the lymph nodes. Of 202 PKH26-GL and Hoechst-stained cells observed, 154 (76%) were positive for the macrophage marker, 32 (16%) for the lymphocyte marker and 16 (8%) were inconclusive.

The notion that HIV-1 transmission is mediated by HIV-1-infected lymphocytes and/or monocytes and macrophages was proposed nearly 10 years ago by Anderson [15] and Levi [16]. Since then, it has been shown that HIV-1-infected mononuclear blood cells are present in both semen and vaginal mucus [17,18]. Even if HIV-1 infection is cell-mediated, however, the mechanism is unclear. One obvious mechanism would be the entrance of an infected cell through breaks in the genital tract epithelium. Direct infection of epithelial cells is another possibility. In this regard, we have demonstrated that HIV-1-infected, T lymphoma cells adhere to epithelia derived from the human cervix *in vitro* [19], and that adherence triggers directional secretion of HIV-1 to the surface of the epithelium. Epithelial cells subsequently take up virus and become productively infected [3,20].

Table 1

Number of double-stained cells in iliac lymph nodes

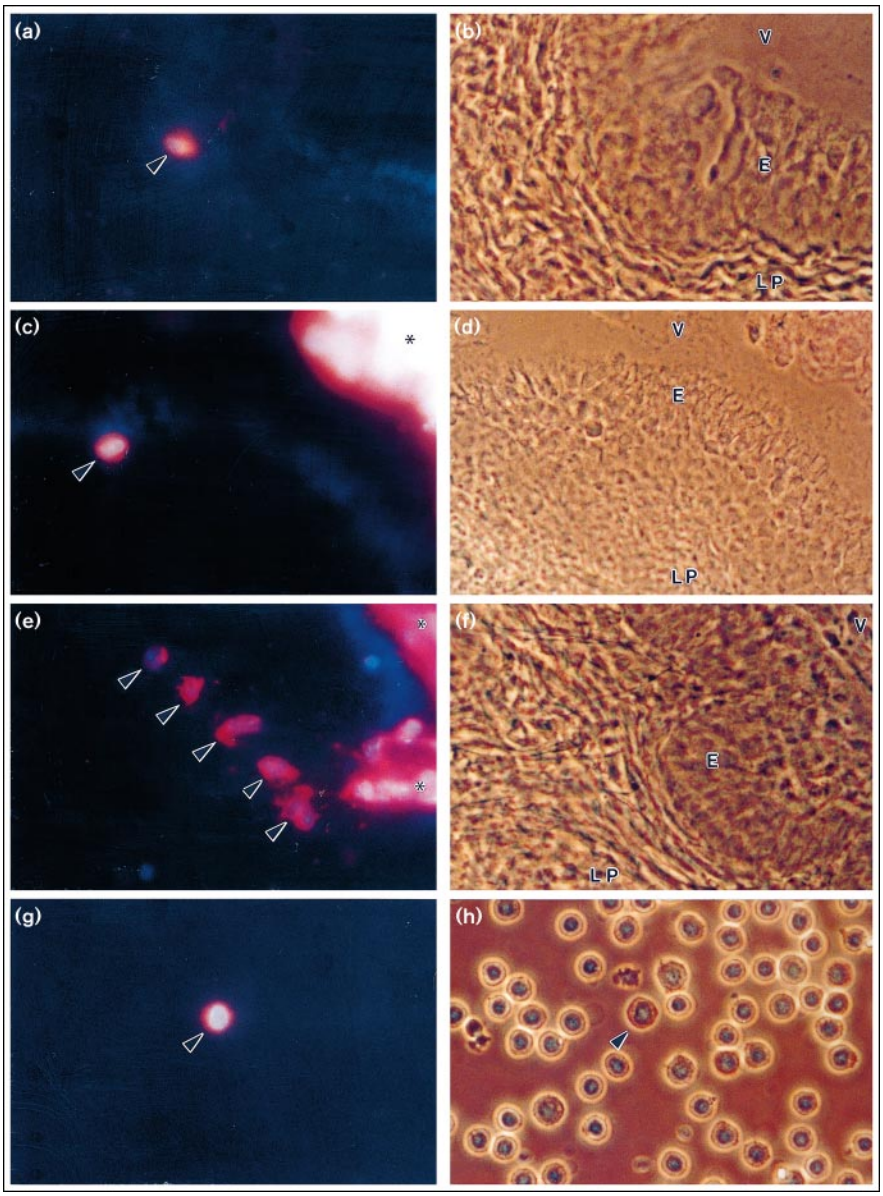
Mouse	Total cells observed	Double-stained cells
1	4.4×10^6	49
2	3×10^6	30
3	5.4×10^6	68
4	1.9×10^6	54
5	4.1×10^6	39
6	4.4×10^6	27
7 (control)	2.7×10^6	0
8 (control)	2.5×10^6	0
9 (control)	3.5×10^6	0
10 (control)	3.5×10^6	0

Cells from dissociated iliac lymph nodes were counted in six experimental and four control mice. Fluorescence microscopy was used to examine all cells with both blue nuclei and red cytoplasm. Mouse numbers 1–4 were BALB/c mice donor and recipient. Recipient animals were inoculated with a pipet tip. Mouse numbers 5,6,9 and 10 were BALB/c donor and recipient outbred CD1; these mice were inoculated with a silastic tube attached to a pipet tip. Controls 7 and 8 were untreated; controls 9 and 10 were inoculated with freeze-thawed double-stained cells.

Another possible infection mechanism involves migration of HIV-1-infected mononuclear cells between cells of intact epithelia. We recently showed that HIV-1-infected, activated primary monocytes typically crawl along surfaces, putting forward a leading pseudopod from which they secrete HIV-1 virions [21]. When added to monolayers of epithelial cells derived from the human cervix, monocytes migrated between epithelial cells, leading us to speculate that cell migration could be involved in HIV-1 transmission [7]. In this paper, we have presented evidence that mononuclear blood cells are also capable of traversing intact epithelia *in vivo*. Immunophenotyping indicated that both macrophages and lymphocytes can reach the lymph nodes. Although more macrophages than lymphocytes reached the lymph nodes, we placed more macrophages then lymphocytes in the vagina, so they may have similar intrinsic capacities for migration.

It is not surprising that mononuclear blood cells are capable of exiting the vaginal vault. Many cells of the immune system migrate through the body to perform their normal functions. Mononuclear, as well as polymorphonuclear, blood cells migrate from the base of the epithelia to

Figure 1



Fluorescent **(a,c,e)** and matching phase contrast **(b,d,f)** photomicrographs of the vaginal vault (V), epithelium (E) and lamina propria (LP) four hours following inoculation of stained peritoneal cells into the vagina of a mouse. The vaginal epithelium is autofluorescent blue. Double-stained cells are observed in the vaginal vault (*) and in the lamina propria (arrow). The last two panels show fluorescent **(g)** and matching phase contrast **(h)** photomicrographs of dissociated cells from an iliac lymph node; a double-stained cell is indicated by the arrow. (Magnification × 600.)

the vaginal vault, uterine lumen [22,23] and intestine [24]. Furthermore, migration of polymorphonuclear and mononuclear blood cells from the apical surface to the base of capillary endothelia is a basic element of the immune response. At this point, however, we can only speculate that cell trafficking is involved in the sexual transmission of HIV-1.

In vivo evidence in support of cell-mediated transmission comes from the observation by Girard *et al.* [25] that HIV was transmitted by a few hundred cells placed on the intact cervix of three chimpanzees. In macaques, however, SIV can be transmitted through an apparently intact epithelia by inoculation of free virus but not by SIV-infected peripheral blood mononuclear cells [13,26]. In our study, we used an artificial system to increase the likelihood of finding cells. To raise our chances of observing the phenomenon, we treated mice with a progestin which is reported to cause thinning of the vaginal epithelium [27,28]. We also placed approximately the same number of mononuclear cells as would be in an entire human ejaculate [29] in the vagina of an animal which weighs about 2000 times less than a human. However, when one considers that relatively few acts of intercourse result in an infection and that theoretically it only takes a single HIV-1-secreting cell to initiate an infection, the hypothesis that

infection is initiated by migration of an HIV-1-infected, activated mononuclear cell seems more plausible.

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